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PHARMACOLOGY AND TOXICOLOGY OF DIFLUNISAL

C.A. STONE, C.G. VAN ARMAN*, V.J. LOTTI, D.H. MINSKER, E.A. RISLEY, W.J. BAGDON, D.L. BOKELMAN, R.D. JENSEN, B. MENDLOWSKI, C.L. TATE, H.M PECK, R.E. ZWICKEY & S.E. McKINNEY

Merck, Sharp and Dohme Research Laboratories, Merck & Co., Inc., West Point, Pennsylvania 19486

1 Diflunisal, a new salicylate, possesses anti-inflammatory, analgesic and antipyretic activity. It was found to be about 7.5–13 times more active than aspirin as an analgesic (Randall–Selitto assay in rats) and as an anti-inflammatory agent (carrageenin foot oedema and adjuvant arthritis in rats).

2 Diflunisal was 1.4 times as active as aspirin as an antipyretic in the rat and about three times as potent in reducing lameness induced by injecting urate crystals into the knee joint of dogs.

3 Diflunisal inhibited adenosine diphosphate, adrenaline and thrombin-induced aggregation of human platelets *in vitro*. The concentrations required were 18–35 times higher than those needed with aspirin.

4 After single doses, both diflunisal and aspirin induced gastric haemorrhages in starved rats. This was evident only after relatively large doses of diflunisal, whereas aspirin induced gastric changes after pharmacologically active amounts.

5 Diflunisal, but not aspirin, after single doses, produced perforations of the small intestine in the rat. The dose levels required were considerably above those that would demonstrate anti-inflammatory and analgesic activity.

6 In oral subacute toxicity studies in rats and dogs of 14 weeks' duration, diflunisal and aspirin produced similar gastric and renal toxicity. Both produced focal oedema, haemorrhage and small ulcers in one or both species. Renal changes were limited to a slight degree of oedema of the papilla in the rat and dog. Diflunisal, but not aspirin, produced intestinal lesions of the small bowel in the rat but not the dog. The changes with either drug were largely limited to the higher levels used (50–200 mg/kg/d).

Introduction

Diflunisal is a new derivative of salicylic acid which in some, but not all, respects resembles aspirin pharmacologically (Figure 1). It differs from aspirin in having a difluorophenyl group and lacks the acetyl function. These differences account for some of the pharmacological, biochemical and metabolic differences between the two compounds, as discussed below. We summarize here certain pharmacological and toxicological attributes of diflunisal compared with aspirin in laboratory animals. A preliminary report of some of these data has appeared elsewhere (Van Arman *et al.*, 1977).

Methods

Pharmacological studies

Carrageenin-induced foot oedema The technique used was as described by Winter *et al.* (1962) and by Van Arman *et al.* (1965). Male Sprague–Dawley rats (150–180 g) received the drugs by stomach tube in 3 ml 0.5% aqueous methylcellulose, either in solution or suspension. Control animals received the vehicle only. One hour later, 0.05 ml 1% carrageenin was injected subcutaneously under the plantar aspect of the right hind foot. The volume of the foot was determined immediately by immersion of the foot into mercury up to a previously marked ink-spot; the volume of mercury displaced was automatically recorded. Three hours later, the foot volume was measured again. The difference between the two volumes was deemed to be the swelling. Drug effects were calculated as percentage inhibition of swelling, taking the swelling of the control group as 100%.

Adjuvant-induced arthritis The method used was as described by Winter & Nuss (1966). Experiments were carried out in male Sprague–Dawley rats (170–190 g initially). Arthritis was induced by injection, subcutaneously into the distal third of the tail, of an adjuvant (a suspension of 0.5 mg heat-killed *M. Butyricum* (Difco) in 0.1 ml light mineral oil). The

* Present address: Wyeth Laboratories, Radnor, Pennsylvania.

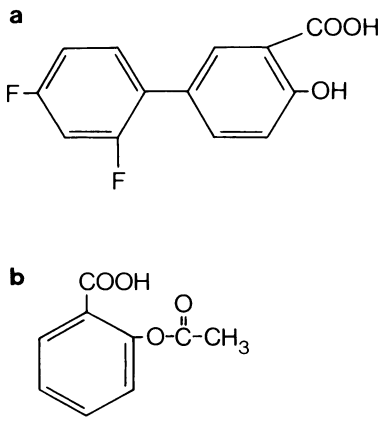

Figure 1 Structure of diflunisal (a) and aspirin (b).

volume of the right hind foot was measured as described above, and individual body weights were recorded. The arthritis was allowed to develop for 14 d; body weights and foot volumes were again determined. Compounds were given by stomach tube, dissolved or suspended in 0.5% aqueous methylcellulose, in such concentration that the volume given was 0.5 ml per 100 g body weight. Control rats received the aqueous methylcellulose solution only. Compounds were given once per day, beginning on the day before (day −1) the single injection of adjuvant on day 0, and continuing every day until day 13. Final measurements were made on day 14.

Antipyretic effects in the rat Male Sprague–Dawley rats (six per treatment group) were injected subcutaneously with 2 ml 7.5% suspension of Brewer's yeast in 0.5% aqueous methylcellulose. An increase in rectal temperature of 2–2.5°C occurred, and this increase is known to persist for more than 24 hours. Eighteen hours after injection, rectal temperatures were recorded and various doses of the test compounds were given orally, suspended or dissolved in 0.5% aqueous methylcellulose. Temperatures were recorded every 30 min for 2 hours. Results are expressed as the mean decrease in rectal temperature (°C). The four differences between the initial reading and each of the four temperature readings were added and divided by 4 to provide mean decrease (°C). The dose–response curve provides the information needed to determine what dose is necessary to induce a change of 1°C in body temperature.

Analgesic activity in rats A method similar to that used here was first described by Randall & Selitto (1975) and modified by Winter & Flataker (1965). One hind foot of a rat was rendered highly sensitive to pressure by injection of an irritant (0.1 ml 5% Brewer's yeast suspended in normal saline) into the subcutaneous tissue beneath the plantar surface of the foot. In certain standard conditions of irritant dosage and timing, the pressure (mm Hg) was determined at which the rat would give a vocal response or 'squeak' when the injected foot was pressed by a plunger. The other hind foot was not injected, and response thresholds were determined for both the inflamed and the normal foot. Two hours were allowed for the oedema and hyperaesthesia to develop before thresholds were determined. Drugs were given to separate groups at such times as to allow determination of thresholds at stated time intervals after dosing. For example, if thresholds were to be determined 5 h after administration, drugs were given 3 h before yeast was injected into the foot. With diflunisal, five separate experiments were carried out at doses of 1, 3 and 9 mg/kg in each experiment, each dose being given to a group of six rats. With aspirin, assays were carried out concurrently with diflunisal; there were two groups of six rats at 6.7 mg/kg, one group each at 10, 30 and 90 mg/kg, four groups each at 20 and 60 mg/kg, and two groups at 180 mg/kg. Injection, order of treatments and determinations of pain reaction threshold, were carried out in random and blind conditions.

Analgesic activity in dogs Sterile sodium urate suspension (as needles, 10–15 μm), 9 mg per 0.45 ml in 0.85% NaCl solution, was injected into one hind knee joint of dogs not previously given an injection of urate and trained to stand quietly for 2.5-min periods on a pivoted board connected to an integrator. Two control readings were taken in the range from 50–100 arbitrary units on the integrator scale. The mean of these readings was considered as 100%, and readings after treatment of the dog were expressed as a percentage of this mean. For more details of the method, see Van Arman & Carlson (1970).

Platelet aggregation studies The method used was as described by Minsker *et al.* (1976). Platelet-rich plasma (PRP) was prepared from citrated blood taken from normal volunteers. Aggregation, measured in a chronologue aggregometer, was induced by the minimal amounts of adenosine diphosphate (ADP), adrenaline and thrombin required to produce both primary and secondary aggregation in control PRP. Diflunisal was dissolved in water by addition of small amounts (0.1–0.5 ml) of saturated lithium carbonate in water. Aspirin was dissolved in saturated sodium acetate and diluted with water as necessary. Aspirin and diflunisal at several different concentrations were added to PRP 20 min before challenge with aggregating agent. Complete absence of the secondary phase of aggregation was used as a measure of inhibition of aggregation and the results expressed as

the percentage of those samples tested that showed no secondary phase.

Toxicological studies

Gastrointestinal effects after single doses The ability of diflunisal and asprin to induce haemorrhage of gastric mucosa and ulcers of the intestine after single doses, was determined using methods described by Brodie *et al.* (1967, 1970). Briefly, gastric effects were studied in groups of male Sprague–Dawley rats (125–150 g) which had been starved for 24 h and then given the drug at various doses, or control vehicle, in 0.5% aqueous methylcellulose. Four hours later, the rats were killed with pentobarbital and the stomachs examined for haemorrhagic areas. Any haemorrhagic area of 2 mm or greater in its largest dimension was considered positive.

The ability of these agents to produce intestinal ulcers of the small bowel was determined in fed male Sprague–Dawley rats (150–200 g). Seventy-two hours after dosing groups of rats with various levels of drug or of the control vehicle, the animals were killed as above and their intestines examined for perforating lesions. The appearance of one perforation, regardless of size, was considered positive.

Acute toxicity The acute toxicity of diflunisal was studied in mice, rats, rabbits and infant and weanling rats. The drug was prepared in various concentrations or suspensions in 1% aqueous methylcellulose. Mice and rats were given the drug by stomach tube using a metal catheter attached to a syringe. Rabbits were given the drug orally using a rubber catheter attached to a syringe; the catheter was flushed with 5 ml of water. Intraperitoneal injections were made in the usual manner.

All animals were observed frequently on the day of drug administration and daily thereafter for 14 days. LD_{50} values were based on 14-d mortality response. Calculations of LD_{50} and the 95% fiducial limits were made using the method of Weil (1952), or the method of Litchfield & Wilcoxon (1949).

A similar acute toxicity study of aspirin was carried out in mice for comparative purposes.

Subacute toxicity A comparative 14-week subacute toxicity study of diflunisal and aspirin was carried out in rats and dogs. Group of 15 male and 15 female Charles River rats were given diflunisal orally by gavage at doses of 12.5, 25, 50 and 100 mg/kg/d, or aspirin at doses of 25, 50, 100 or 200 mg/kg/d for up to 94 consecutive days. Both drugs were suspended in 0.5% aqueous methylcellulose. The rats were 4–5 weeks old when the study began. The groups weighed, on average, 90–92 g (male) and 83–85 g (female). Two similar control groups received the vehicle.

Groups of two male and two female beagle dogs about 11 months old were given diflunisal at doses of 12.5, 25, 50 and 100 mg/kg/d, or aspirin at doses of 25, 50, 100 and 200 mg/kg/d. The drugs were given by gavage in 0.5% aqueous methylcellulose for up to 94 consecutive days. Two similar control groups received the vehicle.

Body weights and physical signs were monitored closely throughout the study period. Ophthalmological and various haematologic examinations were carried out at periodic intervals. Clinical chemical determinations of blood and urine were carried out in the dog. Details of the types and frequency of these and other determinations have been described previously (Zwickey *et al.*, 1974; Peck *et al.*, 1967).

Autopsy examination was carried out in all rats and dogs. Detailed microscopic examinations were carried out on tissues from eight female and eight male rats of one control group and of the group of rats receiving high doses of aspirin and diflunisal. Microscopic examination of sections of the stomach and kidneys was carried out in all rats. Detailed microscopic examination was carried out in all dogs of one control group and in all dogs receiving the highest dose of each drug. Sections of stomach, kidney and adrenals were examined in all dogs. A listing of the tissues examined at the highest doses was similar to that described previously (Zwickey *et al.*, 1974; Peck *et al.*, 1967).

Plasma determinations of diflunisal and salicylate were carried out in certain of the above experiments. Diflunisal levels were measured using the method of Tocco *et al.* (1975). Salicylate was determined using a procedure modified after that described by Chirigos & Udenfriend (1959).

Results

Pharmacological studies

The pharmacological studies have demonstrated that diflunisal possesses the principal pharmacological properties of aspirin and other non-steroidal anti-inflammatory drugs. Thus, it was capable of exerting anti-inflammatory, antipyretic and analgesic activities.

Anti-inflammatory activity of diflunisal was shown by an ability to reduce carrageenin foot oedema in the rat (Table 1). Substantial inhibition was achieved at doses varying from 1.11–30 mg/kg orally. The dose required to cause 50% inhibition, as calculated from these data, was 9.8 mg/kg with confidence limits of 7.1–14.1 mg/kg. Aspirin, studied similarly in this laboratory for several years, at doses of 20, 60 and 100 mg/kg orally in a total of 756 rats, yielded an ED_{50} of 89.2 mg/kg with 95% confidence limits of 81.8–97. Diflunisal is therefore about nine times as potent as aspirin in this assay.

When drug treatment was given 6 h before carrageenin injection, with foot volumes measured 3 h later (total of 9 h after drug administration rather than

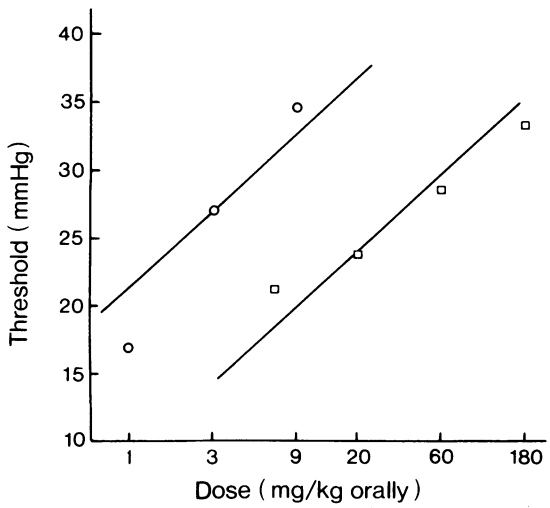

Figure 2 Analgesic effects in the rat against yeast hyperaesthesia. The potency of diflunisal (o,$y=11.8 \log x+21.4$) with respect to aspirin ($y=11.8 \log x+8.7$) was 12.9 (4.4, 39.8). Thresholds were determined 1 hour after administration of the drugs. Tests of validity (and conclusions): preparation (NS); regression (significant); parallelism (NS); curvature (NS).

4 h), the ED_{50} for diflunisal (36 rats) was 9.5 mg/kg orally. The ED_{50} for aspirin in a simultaneous assay (36 rats) was 160 mg/kg orally. Thus, diflunisal possesses a longer duration of action than aspirin.

Diflunisal was also capable of reducing increased foot volume occurring after induction of adjuvant arthritis in the rat. The drug was active at approximately the same levels as those found to be active in reducing carrageenin foot oedema (Table 1). Aspirin was also active in reducing foot volume in adjuvant arthritic rats. The ED_{50} for aspirin, as estimated from a number of assays involving a total of 288 rats, was 78.3 mg/kg. A similar value, estimated for diflunisal from the data in Table 1, was 10.4 mg/kg. Thus, diflunisal is about 7.5 times as potent as aspirin.

The antipyretic activity of diflunisal compared with that of aspirin is shown by the data in Table 2. Both drugs produced a dose-related decrease in rectal temperature. In contrast to their relative activity in the anti-inflammatory assays described above, diflunisal was only slightly more active than aspirin.

The analgesic activity of diflunisal and aspirin was studied in two models, one involving a hyperaesthetic state of the rat foot injected with yeast and the other involving lameness induced by injecting urate crystals into the knee joint of the dog. The relative effectiveness of diflunisal and aspirin to elevate the pain threshold of the hyperaesthetic foot of the rat 1 h after administration of each drug, is shown by the data in Figure 2. As is evident, diflunisal produced more or less similar effects as aspirin but required about 1/13 the amount of drug. Neither drug at the highest level tested, altered the pain threshold of normal feet. This distinguished diflunisal and aspirin from narcotic analgesics, which are well known to elevate pain thresholds of the yeast-injected as well as normal feet.

The duration of analgesic activity of diflunisal and aspirin is illustrated by the data in Table 3. With both drugs analgesic activity was evident 1 h after administration and was well maintained for 4 h; however, the analgesic activity fell precipitously to control levels between the hours 4 and 5. Thus, the duration of analgesic activity of diflunisal in the rat is no longer than that obtained with aspirin. This result is at variance with the duration study using the carrageenin foot oedema assay described above.

Diflunisal and aspirin prevented lameness in dog hind limb induced by the injection of urate crystals

Table 1 Effect of diflunisal on carrageenin-induced foot oedema and adjuvant-induced arthritis

*Procedure**	*Dose (mg/kg orally)*	*No. of rats*	*% Inhibition of increase in foot volume*	*ED_{50} (mg/kg orally)*
Carrageenin-induced foot oedema	1.11	30	27	9.8
	3.33	72	34	
	10.0	78	50	
	30.0	78	65	
Adjuvant-induced arthritis	6	12	35	10.4
	12	12	62	
	24	12	70	

* The carrageenin data were combined from 15 separate experiments involving 43 groups of six rats each. The data in the adjuvant arthritis experiment were derived from two experiments using six rats at each level. The dosage regimen in the carrageenin assay was single dose, whereas that in the adjuvant arthritis procedure involved single daily doses for 14 days. See methods section for other details.

into the knee joint (Table 4). Both drugs exerted similar effects over the 5-h period of observation. Diflunisal was somewhat more potent than aspirin in this test. Both drugs (diflunisal 25 mg/kg orally; aspirin 70 mg/kg orally) also restored mobility to almost normal in dogs that received the drug after lameness had fully developed because of previous injection of urate (data not shown).

Diflunisal and aspirin inhibited aggregation of human platelets *in vitro* (Table 5). Both drugs inhibited secondary aggregation induced by ADP, adrenaline and thrombin, but aspirin was clearly more effective in each instance. Neither aspirin nor diflunisal inhibited the primary phase of aggregation that is evident using ADP as the aggregating agent. It is of interest that the approximate EC_{50} obtained with diflunisal in these

Table 2 Effects of diflunisal and aspirin on rectal temperature of rats with yeast-induced fever*

Drug	*Dose (mg/kg orally)*	*No. of rats*	*Mean decrease in rectal temperature (°C)*	*Effective dose (mg/kg orally)*
Diflunisal	6.25	6	0.21	
	12.5	18	0.36	27.8
	25.0	24	1.03	
	50.0	12	1.31	
Aspirin	25.0	60	0.04	
	50.0	72	1.14	40.2
	100.0	60	1.75	

* Combined results from separate experiments using six rats at each dose. See methods section for other details.
† Dose required to decrease temperature by 1 °C.

Table 3 Duration of analgesic activity of diflunisal and aspirin in rats

Compound	*Dose (mg/kg orally)*	*No. of rats*	*Time after administration (h)*			
			1	*2*	*4*	*5*
			Mean reaction threshold (mm Hg) of inflamed foot			
Control	–	6	11.8	12.5	12.1	12.7
Diflunisal	3.0	6	20.3	20.3	19.8	10.5
	9.0	6	27.3	25.3	25.5	14.3
Aspirin	30.0	6	22.8	18.1	18.8	12.1
	90.0	6	29.6	27.8	17.6	11.5

Table 4 Effect of diflunisal and aspirin on urate-induced lameness in the dog*

Drug	*No. of dogs*	*Dose (mg/kg orally)*	*Mean percentage normal foot pressure after urate injection*			*Approximate ED_{50} at 3 h*
			2 h	*3 h*	*5 h*	
Untreated	7	–	6	3	2	
Diflunisal	4	15	40	46	76	
	4	26	43	38	55	22
	2	45	94	93	93	
Aspirin	2	30	0	0	0	
	4	52	30	10	45	72
	2	90	80	77	93	

* Drugs were given at approximately the same time as the injection of sodium urate crystals into the knee joint of one limb. Foot pressure was measured as described in the methods section.

experiments were generally above those found in plasma of individuals given therapeutic doses (see Tempero *et al.*, 1977).

Toxicological studies

Gastrointestinal effects after single oral doses Most anti-inflammatory drugs are known to produce irritation and/or ulcers of the gastrointestinal tract. In the rat, this is manifested by small haemorrhagic areas in the stomach (generally not ulcer-forming). In addition, some non-steroidal drugs may produce perforating ulcers of the small bowel even after single doses.

In starved rats, both aspirin and diflunisal produced small gastric haemorrhages in the stomach when

Table 5 Effect of diflunisal and aspirin on ADP, adrenaline, and thrombin-induced aggregation of human platelets*

Aggregating agent	*Compound*	*Concentration (μg/ml)*	*Number of samples with no secondary aggregation* / *Number tested*	*Approximate EC_{50} (μg/ml)†*
ADP	Diflunisal	6.25	1/6	
ADP		12.5	1/6	17
ADP		25.0	5/6	
ADP		50.0	5/6	
Adrenaline		50.0	0/4	
Adrenaline		100.0	1/5	140
Adrenaline		200.0	4/5	
Adrenaline		400.0	2/2	
Thrombin		18.7	0/3	
Thrombin		37.5	2/5	50
Thrombin		75.0	6/8	
Thrombin		150.0	5/5	
ADP	Aspirin	0.156	0/4	
ADP		0.625	1/5	0.9
ADP		1.25	3/5	
ADP		2.5	5/5	
Adrenaline		2.0	0/3	
Adrenaline		8.0	4/8	8.0
Adrenaline		32.0	6/6	
Thrombin		0.312	0/6	
Thrombin		1.25	2/7	1.4
Thrombin		5.0	6/6	

* See methods section for details of the procedure.
† Estimated graphically.

Table 6 Acute toxicity of diflunisal in various species

Species	*Sex*	*No. of animals per dose*	*LD_{50} (mg/kg orally) (95% confidence limits)*
Mice (CF_1S)	Female	10	439 (397–496)
Rat (CRCD)	Male, young adult	10	710 (556–906)
Rat (CRCD)	Female, young adult	10	826 (589–1,160)
Rat (CRCD)	Male weanling	10	470 (384–574)
Rat (CRCD)	Female weanling	10	610 (415–898)
Rat (CRCD)	Infant, male and female	10	185 (137–250)
Rabbit (New Zealand)	Random	10	603 (503–788)

examined 4 h after drug administration. The ED_{50} obtained in assays with aspirin using 12 groups (total 62 rats) and nine doses (8–192 mg/kg orally) was calculated to be 81.3 mg/kg (95% confidence limit; 57.1–100.5). In a similar study with diflunisal (seven groups of five rats per dose; three groups of ten rats per dose) over a range of 256–1024 mg/kg orally, an ED_{50} could not be determined because of an appreciable incidence of death at the higher levels. No erosions were, however, obtained at 256 mg/kg; some were evident at 512 mg/kg, but about one-half the rats died at this level.

Diflunisal, but not aspirin, was found to produce perforation of the small intestine after single doses. The ED_{50} for diflunisal, estimated graphically from data obtained from 49 rats, in groups of five or ten, at doses of 64–1024 mg/kg orally, was 520 mg/kg. Aspirin at doses up to 1024 mg/kg orally did not produce intestinal perforations. Diflunisal therefore differs from aspirin in this respect and resembles certain other non-steroids (for example, indomethacin). Note, however, that intestinal ulcer-forming doses of diflunisal were considerably above those exerting anti-inflammatory and analgesic activity in the same species.

Acute toxicity The LD_{50} of diflunisal, determined in a number of species, are listed in Table 6. Signs of toxicity before death were non-specific, consisting of bradypnoea, ataxia, tremors and convulsions. Death in mice and rats occurred generally in less than 100 min or overnight; rabbits died up to 5 d after drug administration. No sex differences were apparent in adult or weanling rats. The infant rat was, however, clearly more susceptible than the older rat, whereas the weanling rat was only slightly more susceptible.

The LD_{50} for aspirin in CF_1S female mice was 1225 (range 1140–1310) mg/kg orally. Thus, diflunisal is about three times more acutely toxic than aspirin in this species. An increased susceptibility of infant animals to aspirin has been described previously (Goldenthal, 1971).

Subacute toxicity studies in rats Ptyalism was seen shortly after dosing in both drug-treated and control animals, especially at the two highest doses of diflunisal and aspirin. Only one death attributable to treatment occurred during the experiment. This rat, given diflunisal 100 mg/kg/d, had marked weight loss, an unkempt coat, excessive micturition, soft stools, weakness, decreased haemoglobin concentration and haematocrit, and leucocytosis before dying in week 11. Autopsy examination of this animal revealed drug-related perforations of the small intestine and peritonitis. Average leucocyte counts higher than control values were observed in female rats given diflunisal 100 mg/kg/d or aspirin 200 mg/kg/d in week 8, and in male and female rats at these doses in

Table 7 Diflunisal and aspirin: 14-week comparative oral toxicity study in rats

Organs	*Lesions*	*Control*	*No. of rats with lesions/Total number treated*								
			Diflunisal (mg/kg/d)				*Aspirin (mg/kg/d)*				
			12.5	*25*	*50*	*100*	*25*	*50*	*100*	*200*
Stomach										
Mucosa	Focal necrosis	1/60	0/30	0/30	0/30	2/30	1/30	1/30	0/30	1/30
	Focal oedema	0/60	0/30	0/30	0/30	0/30	0/30	0/30	1/30	1/30
	Focal haemorrhage	0/60	0/30	0/30	0/30	0/30	0/30	0/30	1/30	2/30
Small intestine	Ulcerative enteritis	0/60	0/30	0/30	0/30	5/30	0/30	0/30	0/30	0/30
Peritoneum	Peritonitis	0/60	0/30	0/30	0/30	0/30	0/30	0/30	0/30	1/30
Kidney	Papillary oedema	0/60	0/30	0/30	0/30	2/30	0/30	0/30	1/30	4/30

week 12. Male rats given aspirin 200 mg/kg/d had average body weights 6–10% less than controls from week 4 until termination; the remaining rats had no weight change. There were no drug-induced changes in the results of the ophthalmological examinations.

Principal findings from post-mortem studies conducted are listed in Table 7. As shown, gastric lesions were found in a small number of rats treated with aspirin at doses of 25, 50, 100 or 200 mg/kg/d, or with diflunisal 100 mg/kg/d. Ulcerative enteritis was also observed in rats treated with diflunisal 100 mg/kg/d. One rat on aspirin 200 mg/kg/d was found to have focal peritonitis, perhaps due to a healed perforated ulcer of the stomach. Renal papillary oedema was observed at low incidence in rats treated with aspirin 100 or 200 mg/kg/d or diflunisal 100 mg/kg/d. Other than ulcerative enteritis, which was sufficiently severe to result in the death of the rat mentioned above, the gastric and renal lesions found with both drugs were focal and very slight in degree. No other drug-related histomorphological alterations were evident in other tissues examined.

Subacute toxicity studies in dogs Emesis and ptyalism occurred at the two highest doses of diflunisal (50 or 100 mg/kg/d) and at the highest dose of aspirin (200 mg/kg/d). Soft stools occurred in dogs given diflunisal 100 mg/kg/d. A slight decrease in heart rate occurred in dogs treated with aspirin 200 mg/kg/d. Decreased food intake for a few days and a transient weight loss occurred in one dog which received aspirin 200 mg/kg/d. All dogs survived the study.

A decrease in average haemoglobin concentration and haematocrit was observed in dogs given diflunisal 100 mg/kg/d or aspirin 200 mg/kg/d in weeks 4, 8 and 12. A neutrophilic leucocytosis and an increased erythrocyte sedimentation rate were observed in one dog that received aspirin 200 mg/kg/d in week 4, but not thereafter. A decrease in serum albumin occurred in all dogs that received diflunisal 100 mg/kg/d and half of the dogs that received diflunisal 50 mg/kg/d, or aspirin 200 mg/kg/d. This was shown to be caused by interference in the chemical determination of albumin. Elevation of serum glutamic-oxalic transaminase activity was observed in one dog given diflunisal 100 mg/kg/d in weeks 4 and 12 of the study. No other biochemical abnormalities were observed.

Plasma concentrations of both drugs were determined at all doses on day 1 of this toxicity study and periodically thereafter. The data obtained at the highest dose of diflunisal and aspirin on day 1 and during week 13 are shown in Table 8. Both agents yielded levels of the same order on day 1. Plasma levels after aspirin tended to be higher after 13 weeks; those after diflunisal were largely unchanged. Tocco *et al.* (1975) have previously presented data showing that diflunisal did not accumulate during the course of this

Table 8 Plasma levels of diflunisal and aspirin after oral administration to beagle dogs

Compound (mg/kg orally)	*Dog No.*	*Plasma concentration (μg/ml)*											
		Drug day 1					*Drug week 13*						
		Time (h) after administration					*Time (h) after administration*						
		0	*1*	*2*	*4*	*6*	*24*	*0*	*1*	*2*	*4*	*6*	*24*
Diflunisal (100)	1 F	0	227	196	158	126	35	9	234	328	234	201	93
	2 F	0	278	264	258	164	36	46	154	159	215	220	51
	3 M	0	128	188	196	196	40	37	146	209	239	229	64
	4 M	0	343	273	143	201	86	85	146	199	278	229	93
	Mean	0	244	230	189	172	49	44	170	224	242	220	75
Aspirin* (200)	1 F	0	124	156	124	95	12	44	415	441	389	345	60
	2 F	0	266	276	269	108	67	81	406	548	450	380	91
	3 M	0	190	208	232	173	34	2	330	759	322	245	52
	4 M	0	196	196	156	142	25	47	160	173	131	102	2
	Mean	0	194	209	195	130	35	44	328	458	323	268	51

* Values for aspirin are in terms of salicylate content.

Table 9 Diflunisal and aspirin: 14-week comparative oral toxicity study in dogs

Organs	*Lesions*	*Control*	*No. of dogs with lesions/total number treated*							
			Diflunisal (mg/kg/d)				*Aspirin (mg/kg/d)*			
			12.5	*25*	*50*	*100*	*25*	*50*	*100*	*200*
Stomach	Ulcer	0/8	0/4	0/4	1/4	2/4	0/4	0/4	3/4	1/4
	Erosion of mucosa	0/8	0/4	0/4	0/4	0/4	0/4	0/4	2/4	2/4
	Oedema of mucosa	0/8	0/4	0/4	1/4	3/4	0/4	0/4	4/4	3/4
	Haemorrhage	0/8	0/4	1/4	0/4	0/4	0/4	0/4	0/4	1/4
Kidney	Papillary oedema	0/8	0/4	0/4	0/4	2/4	0/4	0/4	0/4	1/4

toxicity study as would be expected from an increasing 'steady-state' level measured after chronic dosing. In addition, peak plasma levels achieved in dogs at the highest dose of diflunisal used, were considerably higher than those found in man after single or repeated administration (see Tempero *et al.*, 1977).

Anatomical changes directly attributable to the administration of either diflunisal or aspirin, or both, were observed only in the gastrointestinal tract and kidneys (Table 9). Gastric ulcers and erosions occurred in dogs given diflunisal 50 or 100 mg/kg/d, or aspirin 100 or 200 mg/kg/d. An ulcer was also observed in the colon of one dog given aspirin 200 mg/kg/d. Minor superficial gastric lesions were found in the stomach of one dog given diflunisal 25 mg/kg/d, but were of questionable significance as the lesions were small and circumscribed. The renal lesions noted in Table 9 were all very slight in degree and were restricted to those groups receiving the highest doses of both drugs.

Discussion

The present study has demonstrated that diflunisal, a salicylic acid derivative, possesses many of the pharmacological and toxicological attributes of aspirin. Thus, both compounds show analgesic, antipyretic and anti-inflammatory activities. Diflunisal was nearly ten times more potent than aspirin in anti-inflammatory and analgesic experiments in rats, but only about three times as potent in the urate pain model in the dog and 1.4 times as potent as aspirin as an antipyretic in the rat. Toxicologically, diflunisal also resembled aspirin in that both agents produced gastrointestinal and renal changes in acute and subacute toxicity studies. Diflunisal, however, was distinctly less active than aspirin in producing gastrointestinal changes after single doses.

The principal pharmacological properties of aspirin and other non-steroidal anti-inflammatory agents may be due to their ability to inhibit prostaglandin (PG) synthetase (cyclo-oxygenase). The evidence supporting this hypothesis has been summarized by Ferreira & Vane (1974). Diflunisal also inhibits PG synthetase (Majerus & Stanford, 1977), and, therefore, may involve the same mode of action. As shown by Majerus & Stanford (1977), aspirin covalently acetylated the enzyme and was considerably more active in inhibiting PG synthetase from human platelets than that derived from sheep seminal vesicles. Diflunisal, on the other hand, did not covalently bind with the enzyme and was equally effective in inhibiting the enzyme from both sources. The lack of selectivity for platelet enzymes exhibited by diflunisal may account, at least in part, for its relative inability to inhibit aggregation of human

platelets, as demonstrated in Table 5 and by Smit Sibinga (1977), after oral administration. Note, however, that plasma levels of diflunisal, after administration of therapeutic doses, were generally lower than those needed to inhibit platelet aggregation experiments *in vitro* (Tempero *et al.*, 1977 and Table 5).

Differences in relative potency between aspirin and diflunisal in the various anti-inflammatory, analgesic and antipyretic assays used, may also be related to relative selectivity for the PG synthetases involved in each response. Differences in penetrability to the site of action, however, may be equally, if not more, important. For example, diflunisal is known to be bound considerably (>99%) to rat and human plasma (Tocco *et al.*, 1975), and its penetration into various tissues may vary. Thus, its lesser degree of potency in the dog knee joint assay compared with its activity in the inflamed foot model in the rat may be the result of a lesser degree of penetration. Additional studies relating to this factor, as well as those involving inhibition of the specific PG synthetases involved, are required to understand fully the differences between the two drugs.

As described by van Winzum & Rodda (1977), diflunisal has a relatively long duration of action in humans as an analgesic agent. The data in Table 3 show that its effects in the rat persisted for periods less than 5 hours. The half-life of diflunisal in the rat, however, is appreciably shorter than in human subjects (Tocco *et al.*, 1975), and this probably accounts for the differences between the two species. Note that the peak plasma levels of diflunisal in the rat after 10 mg/kg orally averaged 48 μg/ml (Tocco *et al.*, 1975). As discussed by Tempero *et al.* (1977), peak plasma levels of diflunisal 20–30 μg/ml were found after an analgesic dose of 250 mg to human subjects; thus, as reflected by plasma levels, the two species are about equally sensitive to the agent.

The principal toxicological attributes of diflunisal and aspirin revealed after single or subacute administration involved the gastrointestinal tract and the kidney. The changes in the kidney of the rat and dog were limited to small degrees of oedema of the papilla and were only observed at higher doses. Both agents produced small, superficial haemorrhagic areas in stomachs of starved rats after single doses. From the single dose experiments, however, it was clear that diflunisal produced less apparent irritation of the mucosa than aspirin. Indeed, the doses of aspirin that produced gastric pathology were very close to those required to demonstrate its anti-inflammatory or analgesic actions in the rat. Diflunisal did not produce gastric changes in amounts 25 times greater than doses that exhibited anti-inflammatory and/or analgesic activity.

Gastric pathology was also evident in the rat and dog during the 14-week toxicity study. In the dog, both drugs induced similar gastric pathology at more or less similar doses. In both instances the changes were relatively minor. In rats, focal oedema and haemorrhage of the mucosa were only observed with aspirin in low incidence. Focal mucosal necrosis occurred at a very low incidence in treated and control animals, and is therefore probably spontaneous in origin. Although a greater degree of gastric pathology might have been expected with aspirin from the single dose experiments carried out with starved rats, it is well known that food in the stomach exerts a protective effect from this action of aspirin (Brodie & Chase, 1967). Food was, of course, available *ad libitum* to the animals during the subacute study.

Diflunisal, but not aspirin, produced perforating ulcers of the small bowel in the rat; these were observed after large single doses (>256 mg/kg orally) of diflunisal. Ulcerative enteritis was found after subacute administration of smaller amounts (100 mg/kg/d; Table 7). Similar intestinal lesions have been described with other non-steroidal anti-inflammatory agents (Stone *et al.*, 1974). Dependency, both on dose and duration of exposure, is a characteristic of other non-steroidal anti-inflammatory agents (Stone *et al.*, 1974). For indomethacin, it is known that intestinal lesions in rats depend on the excretion of the active drug in the bile (Brodie *et al.*, 1970; Duggan *et al.*, 1975). As has been shown by Tocco *et al.* (1975), diflunisal was excreted to some extent in the bile in the rat. Thus, the same mechanism may be involved in both diflunisal and indomethacin metabolism. Note, however, that diflunisal is excreted both by way of the kidney and in the faeces in the dog, in which species intestinal lesions were not produced. Human subjects excrete the drug almost exclusively in the urine (Tocco *et al.*, 1975).

Additional long-term toxicity studies on diflunisal have been carried out in the rat and dog. Pathological changes were not prominent in dogs and rats given diflunisal 10–40 mg/kg/d for 26–59 weeks; a small gastric ulcer was observed in one out of four dogs given 40 mg/kg/d for 27 weeks and in one out of six dogs given 20 mg/kg/d for 58 weeks. A low incidence of gastrointestinal ulceration (2 out of 50 rats) given 40 mg/kg/d for 58 weeks was observed; focal gastritis occurred in one rat given 40 mg/kg/d for 27 weeks. Thus, the changes observed in long-term studies are essentially similar to those observed during the 14-week study reported in detail.

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